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β-2-Thienyl-DL-alanine as an Inhibitor of Phenylalanine Hydroxylase and Phenylalanine Intestinal Transport

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The inhibitory properties of β-2-thienyl-DL-alanine on rat phenylalanine hydroxylase from crude liver and kidney homogenates were assessed *in vitro* and *in vivo*, as well as its effects on the intestinal transport of phenylalanine, by using a perfusion procedure *in vivo*. The apparent K_m for liver phenylalanine hydroxylase changed from 0.61 mM in the absence of the inhibitor to 2.70 mM in the presence of 24 mM-β-2-thienyl-DL-alanine, with no significant change in the $V_{max.}$. For kidney the corresponding values were 0.50 and 1.60 mM respectively. A single dose of β-2-thienyl-DL-alanine (2 mmol/kg) failed to inhibit phenylalanine hydroxylase in either organ. Repeated injections during a 4-day period caused a decline of the enzymic activity to about 40% of controls. Intestinal absorption of phenylalanine when perfused at 0.2–2.0 mM concentration was also competitively inhibited by β-2-thienyl-DL-alanine. Its K_i value was estimated at 81 mM. The limited inhibitory effects of β-2-thienyl-DL-alanine towards hepatic phenylalanine hydroxylase and phenylalanine intestinal transport, and its rapid metabolism, as suggested by the small elimination of this compound in the urine and its virtual absence from animal tissues, are factors that restrict its potential usefulness as an inducer of phenylketonuria in rats or as an effective blocker of phenylalanine absorption by the gut.

Substances known to compete with phenylalanine as substrates for hepatic phenylalanine hydroxylase (EC 1.14.16.1) have been used to reproduce phenylketonuria in experimental animals. Among them, DL-*p*-chlorophenylalanine has been applied extensively to that effect (Lipton *et al.*, 1967; Wapnir *et al*, 1970; Longenecker *et al.*, 1970; Berry *et al.*, 1975; Valdivieso *et al.*, 1975). However, since this compound also inhibits brain tryptophan hydroxylase (EC 1.14.16.4), it is considered less than satisfactory for a valid mimicking of human phenylketonuria (Greengard *et al.*, 1976).

Other ring- and chain-substituted derivatives of phenylalanine have merited attention as potential inhibitors of phenylalanine hydroxylase (De Graw *et al.*, 1967; Counsell *et al.*, 1970). β-2-Thienyl-DL-alanine was described as an inhibitor of the intestinal absorption of phenylalanine in monkeys (Lines & Waisman, 1970*a*) and of renal tubular resorption of that amino acid (Lines & Waisman, 1970*b*). It has been found also to be a specific competitor for jejunal phenylalanine transport in rats (Wapnir & Lifshitz, 1974). The present studies were intended to determine whether, in addition to its properties at the membrane transport level, β-2-thienyl-DL-alanine may be effectively used as an inhibitor of hepatic and renal phenylalanine hydroxylase, in experimental animals. In addition, we explored further its action on the intestinal absorption of phenylalanine, as a possible way to block the occurrence of the hyperphenylalaninaemia characteristic of untreated phenylketonuria.

Materials and Methods

Experimental animals

Tissues from male 150 g Wistar-derived rats (Charles River, Wilmington, MA, U.S.A.) were obtained under ether anaesthesia. They were chilled in ice and immediately homogenized in 4 vol. of ice-cold 0.14 M-KCl containing 2.5 mM-NaOH. The homogenates were centrifuged at 750 *g* at 2°C for 15 min to remove debris, and the supernatants further centrifuged at 16000 *g* at 2°C for 20 min. The final supernatant was used for the enzyme assay.

For inhibition experiments *in vivo*, male rats weighing between 60 and 80 g were injected intraperitoneally with 2 mmol of β-2-thienyl-DL-alanine/kg dissolved in 0.1 M-HCl and neutralized just before use with $NaHCO_3$. Control animals were injected with a comparable volume of 0.15 M-NaCl. During the course of the study, the rats had free access to food (Purina Lab Chow, Ralston Purina Co., St. Louis, MO, U.S.A.) and water. At 1 and 24 h after a single injection, or after 4 days of β-2-thienyl-DL-alanine injections given twice daily, the rats were anaesthetized with 1.8 g of urethane/kg. For repeated doses, the last injection was administered 16h before the animals were killed. Blood was drawn from the abdominal aorta, and the liver and kidney were removed and treated as indicated above. During these chronic experiments, the rats were kept in metal metabolic cages prepared for the collection of faeces-free urine. Boric acid crystals were used as a preservative. The animals were kept in a temperature-controlled room with a 12h light cycle.

Chemicals

2-Amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine hydrochloride was obtained from Aldrich Chemical Co., Milwaukee, WI, U.S.A. Dithiothreitol (Cleland's reagent), urethane and β-2-thienyl-DL-alanine were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Poly(ethylene glycol) 4000 was a product of J. T. Baker and Co., Phillipsburg, NJ, U.S.A. Other chemicals were purchased from Fisher Scientific Co., Fair Lawn, NJ, U.S.A. ACS scintillator fluid was from Amersham Corp., Arlington Heights, IL, U.S.A. L-[U-^{3}H]-Phenylalanine was obtained from New England Nuclear Corp., Boston, MA, U.S.A.

Assays

Phenylalanine hydroxylase in liver and kidney was determined by the procedure of McGee *et al.* (1972). For the inhibition experiments *in vitro*, β-2-thienyl-DL-alanine was dissolved at 240mM concentration by adding HCl and dispensing the appropriate amounts to the incubation tubes. The pH of the incubation mixture was re-adjusted by addition of $NaHCO_3$. Since neutralized solutions of β-2-thienyl-DL-alanine tend to form a precipitate on standing, it is possible that during the incubation in the enzyme assay the actual concentration achieved after adding the appropriate amount of β-2-thienyl-DL-alanaine would have been lower than calculated. Protein was assayed by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Phenylalanine and tyrosine concentrations in plasma, tissues and the incubation mixture were determined spectrofluorimetrically (Wong *et al.*, 1964) after deproteinization with an equal volume of 0.6M-trichloroacetic acid. Under the conditions of the assay, β-2-thienyl-DL-alanine produced no fluorescence. The concentration of this compound in plasma, urine and tissues was measured by paper chromatography on Whatman 3MM paper (20cm × 20cm) with an ascending solvent (butan-1-ol/acetic acid/water, 12:3:5, by vol.). A 0.1% ninhydrin solution in acetone containing 2% acetic acid and 0.1% cadmium acetate was used to stain the sheets (Blackburn, 1965). When thus treated, β-2-thienyl-DL-alanine produced a dark purple band, R_F 0.63, well separated from phenylalanine, tyrosine and other major amino acids. The corresponding area was excised and eluted with 1.50ml of methanol overnight and the A_{505} of the resulting colour was determined. Standards were run simultaneously under identical conditions. Under these conditions the lower limit of detection was 0.1mM.

Intestinal perfusions

These were carried out as described fully by Wapnir & Lifshitz (1976). In brief, 150g male rats, previously maintained on Purina Lab Chow, were starved overnight to avoid the presence of chyme in the upper intestinal tract. A 20cm jejunal segment was cannulated under urethane anaesthesia. The rats were then perfused with Krebs–Henseleit (1932) bicarbonate buffer containing 0.2, 0.3, 0.5, 0.7, 1.0 or 2.0mM-L-phenylalanine, with or without 24mM-β-2-thienyl-DL-alanine. Solutions were made iso-osmotic by adjusting the concentration of NaCl. Poly(ethylene glycol) 4000 (mol.wt. 3000–3700) at a concentration of 0.6g/litre was used as a non-absorbable marker. Tracer amounts of L-[U-^{3}H]phenylalanine were added to the buffer. Fractions of the perfused buffer were collected through the distal end of the jejunal segment. After a 45min equilibration period, four samples were collected every 15min for 1h. A second solution was passed immediately after for 90min. During the last 1h, four additional 15min fractions were collected. The samples were centrifuged for 20min at 600*g* to separate cells and debris, and 0.50ml portions of the clear supernatants were transferred to scintillation vials and mixed with 10ml of ACS scintillator fluid. The samples were counted for radioactivity at room temperature (26°C) (Beckman liquid-scintillation system LS-230; Palo Alto, CA, U.S.A.) with an efficiency better than 30%. L-Phenylalanine concentrations were calculated by isotope dilution (Blahd, 1971). Six to eight animals were used for each of the solutions perfused in these experiments. The results were expressed as nmol of L-phenylalanine absorbed by 1cm of intestine/min, after correction for water exchange as indicated from the determination of poly(ethylene glycol) (Malawer & Powell, 1967).

Results

β-2-Thienyl-DL-alanine exerted a marked inhibition *in vitro* on hepatic and renal phenylalanine hydroxylase. Normal Michaelis–Menten kinetics were exhibited by the enzyme in both tissues. In the liver, the K_m in the absence of inhibitor was 0.61mM and increased to 2.70mM in the presence of 24mM-β-2-thienyl-DL-alanine (Fig. 1). Both $V_{max.}$ values were estimated as about 550 units/g of tissue. One unit of activity is the amount of enzyme required to convert 1μmol of substrate/min. For the renal enzyme, the corresponding values were 0.50 and 1.60mM, and the $V_{max.}$ was estimated at 400 units/g (Fig. 2). The K_m values for the enzymes in both tissues were of the same order of magnitude as those of the original method (McGee *et al.*, 1972). Determination of Hill coefficients (Mahler & Cordes, 1971) was carried out from the kinetic data obtained in the assays. For the liver, in the absence and presence of β-2-thienyl-DL-alanine, they were 1.03 and 1.20 respectively, and in the kidney the corresponding values were 0.93 and 1.01.

Dixon (1953) plots were drawn from data of phenylalanine hydroxylase activity when the concentration of the substrate was maintained fixed at 0.3 and 6.0mM for liver preparations, or at 0.2 and 4.0mM for kidney homogenates, while the concentration of β-2-thienyl-DL-alanine was increased over a wide range (Fig. 3). These intercepts yielded K_i values of 3.2 and 3.0mM for liver and kidney respectively.

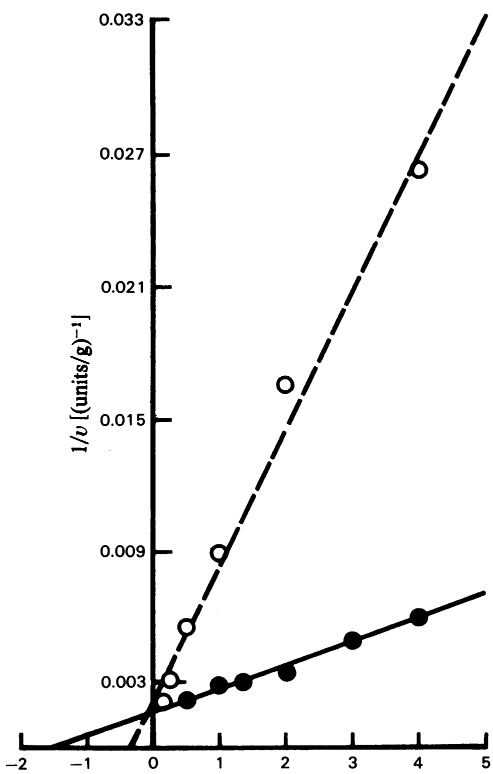

Fig. 1. *Double-reciprocal plot of phenylalanine hydroxylase activity in crude rat liver homogenates, in the absence (●) and in the presence of 24mM-β-2-thienyl-DL-alanine (○)*

Data for the K_m and $V_{max.}$ obtained from regression lines constructed by the least-squares method are indicated in the text.

The administration of repeated doses of β-2-thienyl-DL-alanine over a 4-day period caused a moderate decrease of liver and kidney phenylalanine

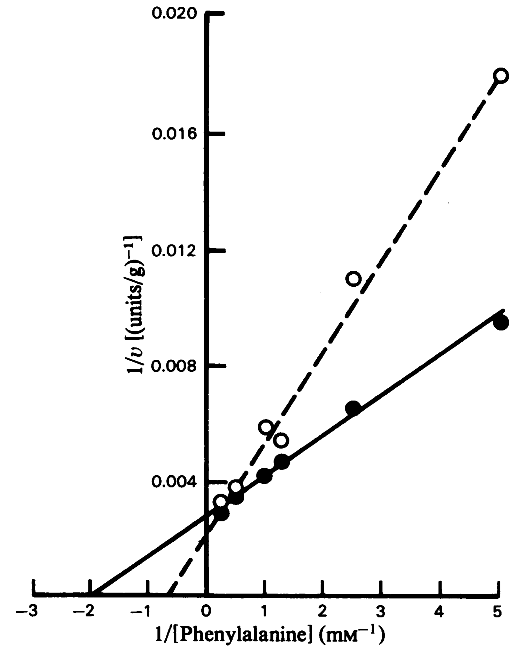

Fig. 2. *Double-reciprocal plot of phenylalanine hydroxylase in rat kidney crude homogenates*

The same captions and units as in Fig. 1 apply here. Calculated data for K_m and $V_{max.}$ are in the Results section.

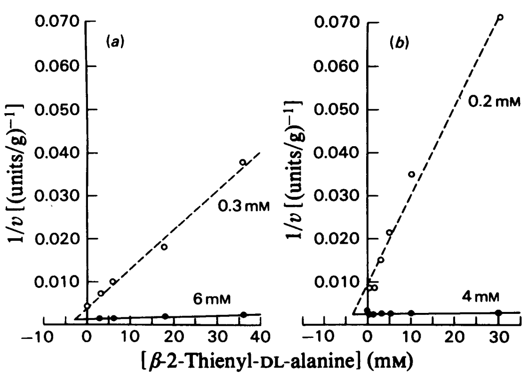

Fig. 3. *Dixon plots of the phenylalanine hydroxylase activity for two concentrations of phenylalanine as indicated in the graphs*

(*a*) Liver; (*b*) kidney. Enzyme determinations were carried out in the presence of increasing concentrations of the inhibitor, β-2-thienyl-DL-alanine, as indicated on the abscissae.

hydroxylase activity to values about 40% of the corresponding controls (Table 1). Single doses of the inhibitor failed to alter the enzyme activity, when measured either 1 or 24h after injection. Immediately after a single administration of β-2-thienyl-DL-alanine, plasma phenylalanine concentration fell below those of control animals. However, β-2-thienyl-DL-alanine had no effect on plasma tyrosine concentration. The liver of rats receiving repeated doses of β-2-thienyl-DL-alanine showed an accumulation of free phenylalanine, whereas the small concomitant decrease in tissue tyrosine concentration was not significant. Although kidney phenylalanine was not altered by any of the treatments, there were minor decreases in the tyrosine content of that organ.

β-2-Thienyl-DL-alanine concentrations were below detectable values in the plasma, liver and kidney of the rats at the times they were killed. The amount excreted in the urine was also minimal. It ranged between 1 and 12% of the dose injected during the previous 24h.

The intestinal perfusions *in vivo* of L-phenylalanine at concentrations ranging between 0.2 and 2.0mM in the absence or presence of 24mM-β-2-thienyl-DL-alanine showed that this compound exerted a moderate inhibitory effect on the intestinal absorption of L-phenylalanine. Increasing concentration of this amino acid neutralized the effect of the antagonist, supporting the concept of a competitive type of inhibition on the mechanism of transport across the small-intestinal mucosa (Fig. 4). In the absence of the inhibitor, we obtained a K_t (transport equivalent of K_m) of 5.1mM. However, after addition of 24mM-β-2-thienyl-DL-alanine, the K_t increased to 39.2mM. The apparent K_i for β-2-thienyl-DL-alanine was calculated from a Hunter & Downs plot (Dixon & Webb, 1964) to be 81mM. The positive slope of the regression line also indicated competitive inhibition.

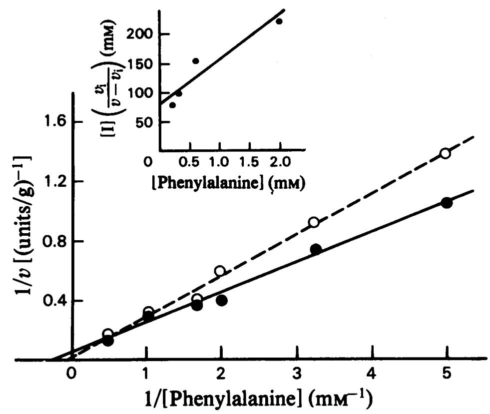

Fig. 4. *Kinetics of the intestinal absorption of phenylalanine in vivo (see the Materials and Methods section), in the absence (●) and in the presence of 24mM-β-2-thienyl-DL-alanine* (○)

The inset represents a Hunter & Downs plot (Dixon & Webb, 1964) that was drawn with the data of the double-reciprocal plot.

Discussion

β-2-Thienyl-DL-alanine is a moderate inhibitor of liver and kidney phenylalanine hydroxylase *in vitro*. For the hepatic enzyme, a 24:1 ratio of the inhibitor to the substrate (24 and 1mM respectively) produced a 72% decrease in the enzyme activity. In kidney preparations, a 20:1 ratio (4 and 0.2mM respectively) resulted in a 48% decrease in the assayable activity of the enzyme. The kinetic data obtained made it apparent that, as could be expected from structural similarities, β-2-thienyl-DL-alanine functioned *in vitro* as a competitive inhibitor of the substrate phenylalanine. As indicated by Hill coefficients close to 1, in neither case, however, could it be demon-

Table 1. *Effects of β-2-thienyl-DL-alanine in vivo*

Data are presented as the means±S.D. for six rats in each group. *$P<0.05$; **$P<0.02$; ***$P<0.01$; ****$P<0.001$; compared with controls by Student's *t* test (Bishop, 1967).

	Treatment			
	One dose		Repeated doses	Controls
	After 1h	After 24h	After 4 days	(0.9% NaCl)
Liver phenylalanine hydroxylase (units/mg of protein)	3.65±0.90	3.73±0.66	1.48±0.30****	3.71±0.66
Kidney phenylalanine hydroxylase (units/mg of protein)	1.54±0.56	1.04±0.23	0.55±0.06****	1.27±0.30
Plasma phenylalanine (mg/dl)	0.79±0.23***	1.78±0.40	2.21±1.20	1.46±0.46
Plasma tyrosine (mg/dl)	1.82±0.85	1.60±0.57	1.66±0.28	1.50±0.23
Liver phenylalanine (μg/g)	14.4±6.5	15.4±9.2	22.6±6.7**	11.1±6.6
Liver tyrosine (μg/g)	10.2±4.4	9.0±3.0	6.9±5.8	8.9±3.7
Kidney phenylalanine (μg/g)	47.6±29.0	43.5±13.0	42.8±6.1	43.4±5.0
Kidney tyrosine (μg/g)	68.9±15.0*	90.0±10.0	78.4±19.5	93.8±20.5

strated that the hepatic or renal enzyme showed co-operative effects; that is, they conformed to simple Michaelis–Menten kinetics.

The saturation kinetics observed for both tissues and comparable K_m values and K_i values for competitors with similar structure such as β-2-thienyl-DL-alanine supported findings obtained under different conditions on the identity of proteins with phenylalanine hydroxylase activity from different organs of man and experimental animals (Berry *et al.*, 1972; McGee *et al.*, 1972; Murthy & Berry, 1974; Ayling *et al.*, 1974, 1975).

In the rhesus monkey, it was observed that β-2-thienyl-DL-alanine was rapidly excreted by the kidney (Lines & Waisman, 1970*a*,*b*). However, for the rat this does not appear to be the case. The absence of significant amounts of free β-2-thienyl-DL-alanine in the liver, kidney or blood therefore suggests that the compound is rapidly metabolized. It has been shown previously (Godin & Gajda, 1967) that phenylalanine and the inhibitor used in the present experiments compete for incorporation into rat tissue proteins. If such were the case, insufficient free β-2-thienyl-DL-alanine would remain to be a competitor for the natural substrate of phenylalanine hydroxylase. Nevertheless, the synthesis of the enzyme *de novo* over a 4-day period could be affected and thus explain the decreased hepatic and renal phenylalanine hydroxylase activity observed in the present studies. The decrease in plasma phenylalanine concentrations measured 1h after injection may be linked to changes in the equilibrium between extra- and intra-cellular amino acid concentrations after the abrupt influx of the phenylalanine analogue.

The intestinal transport of large neutral amino acids is considered to be effected by both common and specific carriers. The proportion of each active transport mechanism has not been clearly defined and may vary with the identity and concentrations of the substrates available to the area of the microvilli where the active sites are considered to be localized (Wapnir *et al.*, 1972; Schultz & Frizzell, 1975; Davenport, 1977). Previous studies *in vitro* and *in vivo* have shown interactions of other amino acids with L-phenylalanine at the level of the small-intestinal mucosa (Schultz & Markscheid-Kaspi, 1971; Wapnir & Lifshitz, 1974). These experiments suggested that an amino acid such as L-alanine would occupy sites in carrier mechanisms common to most neutral amino acids, whereas β-2-thienyl-DL-alanine would pre-empt sites of a specific amino acid carrier. The present study, by providing evidence of a competitive interaction between phenylalanine and β-2-thienyl-DL-alanine, therefore gives further support for the existence of a specific phenylalanine transport system.

A comparison of the enzyme kinetics of both liver and kidney phenylalanine hydroxylase in the absence or presence of a competitive inhibitor, β-2-thienyl-DL-alanine, with a conceptually comparable mechanism, the active transport across the small-intestinal mucosa, indicates that in both phenomena the ratio of the K_i for the inhibitor to the K_m value for the natural amino acid, L-phenylalanine, is roughly of the same order of magnitude. Whereas for hepatic and kidney phenylalanine hydroxylase the K_i/K_m ratio was 5.2 and 6.0 respectively, for experiments *in vitro*, in terms of intestinal transport, the K_i/K_t quotient was 15.8. These data discourage attempts to decrease the intestinal absorption of an individual amino acid by competition for active transport sites by other substances.

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References

Ayling, J. E., Pirson, W. D., Al-Janabi, J. M. & Helfand, G. D. (1974) *Biochemistry* **13**, 78–85

Ayling, J. E., Helfand, G. D. & Pirson, W. D. (1975) *Enzyme* **20**, 6–19

Berry, H. K., Cripps, R., Nichols, K., McCandles, D. & Harper, C. (1972) *Biochim. Biophys. Acta* **261**, 315–320

Berry, H. K., Butcher, R. E., Kazmaier, K. J. & Poncet, I. B. (1975) *Biol. Neonate* **26**, 88–101

Bishop, O. N. (1967) *Statistics for Biology*, pp. 37–45, Houghton Mifflin, New York

Blackburn, S. (1965) *Methods Biochem. Anal.* **13**, 1–45

Blahd, W. H. (1971) *Nuclear Medicine*, 2nd edn., pp. 574–582, McGraw-Hill, New York

Counsell, R. E., Desai, P., Smith, T. D., Chan, P. S., Weinhold, P. A., Rethy, V. B. & Burke, D. (1970) *J. Med. Chem.* **13**, 1040–1042

Davenport, H. W. (1977) *Physiology of the Digestive Tract*, 4th edn., pp. 227–228, Year Book Medical Publishers, New York

De Graw, J. I., Cory, M., Skinner, W. A., Theisen, M. C. & Mitoma, C. (1967) *J. Med. Chem.* **10**, 64–66

Dixon, M. (1953) *Biochem. J.* **55**, 170–171

Dixon, M. & Webb, E. C. (1964) *Enzymes*, 2nd edn, pp. 315–335, Academic Press, New York

Godin, C. & Gajda, A. T. (1967) *Can. J. Biochem.* **45**, 745–747

Greengard, O., Yoss, M. S. & Del Valle, J. A. (1976) *Science* **192**, 1007–1008

Krebs, H. A. & Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33–66

Lines, D. R. & Waisman, H. A. (1970*a*) *Proc. Soc. Exp. Biol. Med.* **135**, 859–863

Lines, D. R. & Waisman, H. A. (1970*b*) *Proc. Soc. Exp. Biol. Med.* **135**, 1061–1064

Lipton, M. A., Gordon, R., Guroff, G. & Udenfriend, S. (1967) *Science* **156**, 248–250

Longenecker, J. B., Reed, P. B., Lo, G. S., Chang, D. Y., Nasby, M. W., White, M. N. & Ide, S. (1970) *Nutr. Rep. Int.* **1**, 105–112

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Mahler, H. R. & Cordes, E. H. (1971) *Biological Chemistry*, 2nd edn., pp. 299–309, Harper and Row, New York

Malawer, S. J. & Powell, D. W. (1967) *Gastroenterology* **53**, 250–256

McGee, M. M., Greengard, O. & Knox, W. E. (1972) *Biochem. J.* **127**, 669–674

Murthy, L. I. & Berry, H. K. (1974) *Arch. Biochem. Biophys.* **163**, 225–230

Schultz, S. G. & Frizzell, R. A. (1975) in *Intestinal Absorption and Malabsorption* (Csaky, T. Z., ed.), pp. 77–93, Raven Press, New York

Schultz, S. G. & Markscheid-Kaspi, L. (1971) *Biochim. Biophys. Acta* **241**, 857–860

Valdivieso, F., Gimenez, C. & Mayor, F. (1975) *Biochem. Med.* **12**, 72–78

Wapnir, R. A. & Lifshitz, F. (1974) *Biochem. Med.* **11**, 370–375

Wapnir, R. A. & Lifshitz, F. (1976) *Proc. Soc. Exp. Biol. Med.* **152**, 307–311

Wapnir, R. A., Hawkins, R. L., Stevenson, J. H. & Bessman, S. P. (1970) *Biochem. Med.* **3**, 397–403

Wapnir, R. A., Hawkins, R. L. & Lifshitz, F. (1972) *Am. J. Physiol.* **223**, 788–793

Wong, P. W. K., O'Flynn, M. E. & Inouye, T. (1964) *Clin. Chem.* **10**, 1098–1104